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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. Serial No.: 09/806,955 Group Art Unit: 1644

Filed: 07/11/2001 Examiner: Jamroz, M.

Applicants: Panayi et al. Attorney Docket No.: 78104.023

Title: **TREATMENT OF INFLAMMATORY DISEASE**

DECLARATION OF GABRIEL S. PANAYI UNDER RULE 132

BOX: FEE
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Gabriel S. Panayi, do hereby declare as follows:

1. I am a co-inventor of the subject matter described and claimed in the above-identified patent application. I am an inventor of each of the claims pending in the application. As such, I am intimately familiar with the contents of the application.

2. A copy of my *curriculum vitae* is attached hereto as Exhibit A, and incorporated herein by reference. As noted in Exhibit A, I hold B.A, M.B, M.D., and D.Sc. degrees from the University of Cambridge. I am currently employed as a research professor, engaged in rheumatology research, at Guy's Hospital, London, England. I am a Fellow of the Royal College of Physicians.

22-7-02

3. The following experiments were performed personally by me, or under my direct control and supervision. The first experiment I performed demonstrates that BiP(GRP78) stimulates secretion of interleukin-10 from human peripheral blood mononuclear cells, while simultaneously suppressing secretion of tumor necrosis factor-α (TNFα) from the same cells.. In this experiment, peripheral blood

mononuclear cells were separated from heparinized blood by density gradient centrifugation. The cells were then prepared at a concentration of 10^6 cells per ml per well in 24-well plates. The cells were then incubated alone (medium only) or in the presence of 20 μg/ml BiP(GRP78), as shown in SEQ. ID. NO: 1 of the patent application. Supernatants were collected at 4, 7, 24, 48, 72, and 96 hours, separated into aliquots, and promptly frozen at -70°C until tested further. Aliquots were then thawed on ice and tested for the presence and concentration of interleukin-10 and TNFα using a commercial ELISA kit (Pharmingen, a division of BD Biosciences, San Diego, CA) and following the manufacturer's instructions. The results are depicted graphically in Exhibit B, attached hereto and incorporated herein by reference. As can be seen from the graph shown in Exhibit B, BiP(GRP78) stimulates secretion of interleukin-10 from human peripheral blood mononuclear cells, while simultaneously suppressing secretion of tumor necrosis factor-α (TNFα) from these cells.

4. The second experiment I performed demonstrates that recombinant human BiP(GRP78) also stimulates interleukin-10 production in mouse mononuclear cells. In other words, the cellular functionality of the recombinant human BiP(GRP78) is conserved in the mouse. In the first part of this experiment, flow cytometry was used to measure intracellular IL-10 in $CD4^+$ cells, using a cytokine secretion assay (Miltenyi Biotech, Auburn, CA). In the second part of this experiment, a commercial cell-based ELISA kit was used to quantify the IL-10 secreted by murine $CD4^+$ cells. DBA/1 mice were immunized with 200 μg recombinant human BiP(GRP78) as shown in SEQ. ID. NO: 1. After 14 days post-inoculation, spleen and lymph nodes were removed. Single cell cultures were prepared and cultured in the presence of human BiP(GRP78) (20 μg/ml) or left unstimulated as a control. For flow cytometry, the cells were incubated for 24 hours. The results are shown in Exhibit C, part 1, attached hereto and incorporated herein by reference. The upper data plot shows the results from the non-BiP-stimulated samples, the lower data plot shows the results from the BiP-stimulated samples. Addressing the top plot: the upper-left and upper-right quadrants show no

cells positive for interleukin-10/phycerythrin (IL-10/PE). The lower-right quadrant shows cells positive for CD4/FITC. Thus, the upper graph clearly shows that the unstimulated cells do not produce IL-10. In short hand, both CD^{+} and $CD4^{-}$ cells are $IL\text{-}10^{-}$. The lower data plot shows the results from the murine cells stimulated with recombinant human BiP(GRP78). Here, the upper-left and upper-right quadrants show cells that have intracellular IL-10. Specifically, the upper-left quadrant of the lower data plot shows that 3.2% of the cells are $CD4^{-}/IL\text{-}10^{+}$. The upper-right quadrant of this same data plot shows that 1% of the cells are $CD4^{+}/IL\text{-}10^{+}$. Although not a terribly high percentage of the cells are positive compared to the negative controls (normal for this type of experiment), these results show that there is a distinct population of murine $IL\text{-}10^{+}$ cells produced by exposure to human BiP. Experiment C, part 2, shows the results of the quantification of IL-10 production following 5 days incubation of murine CD4+ cells either unstimulated or stimulated with recombinant human BiP(GRP78) (SEQ. ID. NO: 1) or with recombinant bacterial heat shock protein 65 as a protein control. As shown in Exhibit C, part 2, attached hereto, there is increased production of IL-10 by CD4+ cells which have recognized BiP(GRP78) from the *in vivo* treatment of the mouse prior to sacrifice and separation of the T cells from spleen and lymph node. This confirms that a distinct population of murine cells are capable of recognizing recombinant human BiP(GRP78).

4 [illegible]
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5. The significance of these three experiments taken in conjunction is that they demonstrate, using objective scientific tests, that recombinant human BiP(GRP78) protein taken from humans exhibits the same biological activity in the mouse. This conservation of biological activity across two very distinct mammalian species indicates that the BiP(GRP78) is likely highly conserved both structurally and functionally across all mammalian species.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this Rule 132 Declaration is directed.

22-7-02.

Gabriel S. Panayi Date

CURRICULUM VITAE

Professor Gabriel S Panayi, ScD, MD, FRCP
Arthritis Research Campaign Professor of Rheumatology
Department of Rheumatology
Guy's, King's and St Thomas' School of Medicine
King's College London,
Guy's Hospital
London SE1 9RT

Qualifications

1962	University of Cambridge, BA (Hons)
1965	University of Cambridge, MB
1971	MD, University of Cambridge
1972	Member of Royal College of Physicians
1981	Fellow of the Royal College of Physicians
1992	Doctor of Science, University of Cambridge

Appointments

1965-1966	House Physician, Queen Elizabeth II Hospital, Welwyn Garden City, Herts
1966 (Feb-July)	House Surgeon, St Mary's Hospital, London W2
1966 (Aug-Sept)	Senior House Officer in Medicine, The General Hospital, Nottingham
1967 (Jan-June)	Senior House Officer in Pathology, Central Middlesex Hospital, London, NW10
1967-1969	Medical Research Council Junior Research Fellow Under Dr DC Dumonde at the Wright-Fleming Institute, St Mary's Hospital Medical School, London, and at the Kennedy Institute of Rheumatology, Bute Gardens, London
1970-1973	Clinical Research Fellow, Arthritis and Rheumatism Council, under Professor JJR Duthie at the Rheumatic Diseases Unit, Northern General Hospital, Edinburgh
1973-1976	Lecturer in Rheumatology, Department of Medicine, Guy's Hospital, London
1976-1980	Arthritis and Rheumatism Council Senior Lecturer and Consultant in Rheumatology, Department of Medicine, Guy's Hospital, London
1980 to present	Arthritis Research Campaign Professor of Rheumatology GKT School of Medicine, Guy's Hospital, London, SE1 9RT

Clinical Service, Management and Leadership

National

1978-1980 Royal Society of Medicine Honorary Secretary, Section of Experimental Medicine
1980-1983 Research Sub-committee, Arthritis Research Campaign
1996-1998 President, Section of Clinical Immunology and Allergy
1980-1982 Member, Executive Committee Heberden Society
1997-1999 British Society of Rheumatology, Member Heberden Committee
1997-1999 Member, Council, British Rheumatology Society
2000-2002 President, British Society of Rheumatology
1997-2002 Research Sub-committee, Arthritis Rsearch Campaign
1997-2002 Oliver Bird Fund, Nuffield Foundation

International

1981 Founder, annual European Rheumatology Research Workshop; premier European immuno-rheumatology meeting. Member of Organising Committee. Organised 1980 (Guy's Hospital) and 2000 meetings (St Catherine's College, Oxford.)
1995- Member European League against Rheumatism, Committee for Investigative Rheumatology
1995- European League against Rheumatism, Committee for Drug Therapy
1995- Member,International League against Rheumatism, Consensus Meeting on Characterisation of Arhroscopy and Synovitis in Rheumatic Diseases
1999-Present European Union of Medical Specialties (Rheumatology)
1997-1999 Organising Scientific Committee, EULAR Congress of Rheumatology, Glasgow

Research activities

Professor Panayi's main research activities are focussed on the following - the pathogenesis of rheumatoid arthritis, the immunotherapy of rheumatoid arthritis, genetics of the rheumatic diseases.

His work has made notable contributions to various aspects of Rheumatology, amongst which may be included - the genetic basis of rheumatoid arthritis, the central role of CD4 positive T-lymphocytes in the pathogenesis, the characterisation of BiP as an autoantigen in rheumatoid arthritis, first use of monoclonal antibodies for the therapy of rheumatoid arthritis.

Professor Panayi is currently interested in the fields of pathogenesis of rheumatoid arthritis and immunotherapy. With regard to the former he, in collaboration with other members of the Department of Rheumatology, has been investigating the role of BiP as an autoantigen in rheumatoid arthritis. BiP is the 70 kD endoplasmic reticulum chaperone. The Department has recently shown that T cells from the synovial fluid from patients with rheumatoid are preferentially stimulated to proliferate.

The second major interest is that of immunotherapy and particularly the use of monoclonal antibodies, particularly directed against T cells, such as anti-CD4 antibodies, to reduce or abolish inflammation in patients with rheumatoid arthritis.

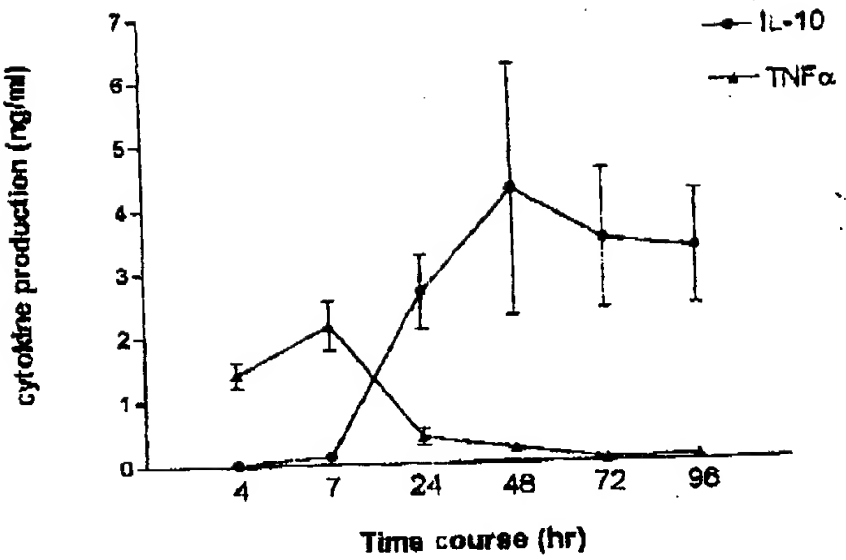
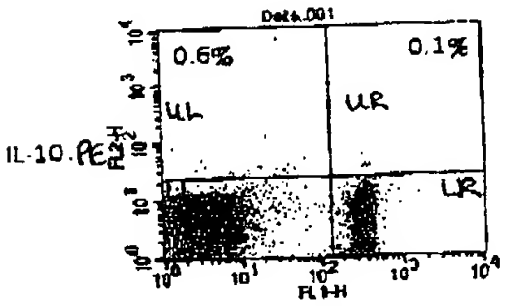


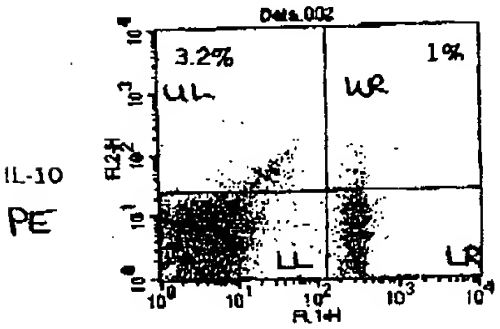
Figure 1. Peripheral blood mononuclear cells (10^6/ml) stimulated by BiP (20µg/ml) over a time course from 4 hours to 96 hours. Results are shown as mean ± standard deviation of 3 experiments

Peripheral blood mononuclear cells were separated from heparinised blood by density centrifugation over Lymphoprep.
Cells were prepared at 10^6 / ml / well in 24 well plates and cultured either alone or with BiP (20µg/ml)
Supernatants were collected at 4, 7, 24, 48 72 96h and aliquoted and frozen and at -70°C until required.
ELISA for IL-10 and TNFα were carried out according to the manufacturers instructions.

BiP Immunised DBA-1 mouse: IL-10 secretion assay



non-stimulated culture



BiP- stimulated culture

FITC - fluorescein isothiocyanate
PE - phycoerythrin.

Figure 2A. DBA-1 mice were immunized with 200µg of BiP, 14 days later spleen and lymph nodes were removed and set up in culture in the presence of BiP (20µg/ml) or left blank as a control for 24 hours. IL-10 secretion from CD4+ T cells was assessed using the Cytokine secretion assay (Miltenyi Biotec) and analysed using flow cytometry. **It is evident that CD4+ cells and other cell types produce IL-10 on stimulation with BiP.**

FACScan dots plots show the fluorescence of CD4+ve T cells (top: upper left (UL) and upper right (UR) no cells positive for IL-10.PE. Lower left (LL) negative cells, lower rt (LR) cells positive for CD4.FITC.)
In the stimulated culture shown below.
UL shows presence of 3.2% CD4⁻ IL-10+ cells (probably mac cyt
UR shows " " " 10+ cells
(although although this is a [illegible] pared with the negative control above there is a distinc[illegible]+ CD4+ cells)
LL - negative cells LR [illegible] cells.

IL-10 production by purified T-cells derived from naive or BiP immunised animals upon restimulation with BiP in culture

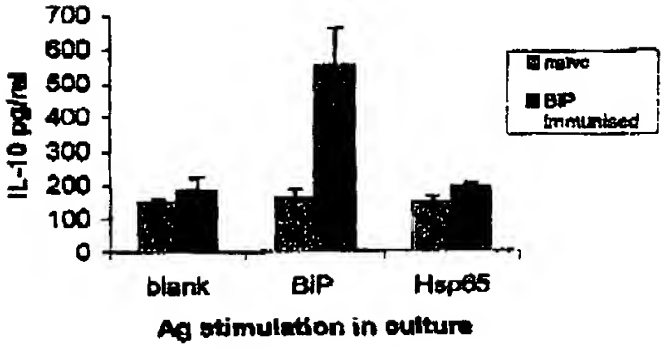


Figure 2B DBA-1 mice (n=5 per group) were immunised with 200μg BiP (naive mice were used as controls). 14 days later T cells were purified (>75% purity as assessed by FACS) from spleens and lymph nodes and mixed with irradiated APC's in a T-cell : APC ratio of 3:1. Cultures were set up in the presence of BiP (20μg/ml), Hsp65 (10μg/ml) as a heat shock protein control, or left blank. IL-10 was measured by CellELISA.
Resting T cells derived from BiP immunised mice secrete low levels of IL-10 (~30pg/ml). However, upon restimulation of cells with BiP in culture for 5days high levels of IL-10 were secreted (400-650pg/ml).

This expt was carried out in mice. At 14days mice were sacrificed and spleens / lymph nodes removed and processed to obtain single cell cultures. Immunomagnetic beads are then used to obtain a negatively selected population of T cells. (This can be done using CD14, CD20, CD56 coated immunomagnetic beads which bind to monocytes, B, NK cells respectively leaving T cells in solution. Beads removed with a magnet). APC will be mixed cell culture required to present antigen but irradiated so no proliferative response and little cytokines released (see blank control)
HSP 65 used a protein control.

Ex. C-2